

**CLEAN VERSION OF REPLACEMENT PARAGRAPHS IN THE SPECIFICATION**  
**PURSUANT TO 37 C.F.R. §121(b) AND OF REWRITTEN, ADDED, AND/OR**  
**CANCELLED CLAIMS PURSUANT TO 37 C.F.R. §1.121 (c)(1)(i)**

**IN THE SPECIFICATION:**

On page 5, lines 10-17, please replace the paragraph with the following:

*B1*  
The present invention encompasses methods for evolving gram positive and gram negative microorganisms as well as yeast, fungus and eucaryotic cells including hybridomas. In one embodiment, the gram negative microorganism includes members of *Enterobacteriaceae* and in another embodiment comprises *Eschericia* and in another embodiment comprises *E.coli* and *E.blattae*. In further embodiments of the present invention, the evolved microorganism includes *E.coli* having ATCC accession number PTA-91 and *E.blattae* having ATCC accession number PTA-92.

Please replace the table on page 6, lines 10-13, with the following rewritten table:

*B2*

Depositor Identification Reference	International Depository Designation	Date of Deposit
<i>Escherichia coli</i> MM294 derivative	ATCC PTA-91	May 19, 1999
<i>Escherichia blattae</i> 33429 derivative	ATCC PTA-92	May 19, 1999

On page 11, please amend the paragraph at lines 1-19, as follows:

*B3*  
The use of a plasmid comprising a mutator gene, ie, a mutator plasmid, can be used to control the mutation rate of a microorganism. As described under Section II below, plasmid constructs can be designed which provide reduced levels of expression of a mutator gene thereby providing a means for altering the ratio of naturally occurring DNA repair genes vs mutator genes. This provides a means for combining the advantage of mutD mutations (which results in random mutagenesis) with the advantages of the other known mutators (lower

*B3*  
*CONT.*

mutation frequency which leads to a lower burden on the cells). Additionally, plasmid constructs can be designed that allow for curing the evolved microorganism of the mutator gene, such as the use of a temperature sensitive origin, thereby allowing for a means for turning the mutation events off and on in the microorganism. For a gram positive microorganism, such as *B. subtilis* where the entire genome has been sequenced, the present invention could encompass the steps of deleting or mutating a DNA repair gene, evolving the *Bacillus* [Bacillus], and restoring the naturally occurring DNA repair system through recombination events. As disclosed herein, several members of *Escherichia*, such as *E. coli* and *E. blattae* have been subjected to the directed evolution methods. Illustrative examples of evolved *E. coli* and *E. blattae* have been deposited with the ATCC and have accession numbers PTA-91 and PTA-92, respectively.

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Please replace the paragraph at page 26, lines 5-15, with the following rewritten paragraph:

*B4*

The evolution of 1,3-propanediol resistance was faster in the presence of B12. After 2 months of evolution GEB025 (+B12) was able to grow with 95-100g/l 1,3-propanediol. After 3 months of anaerobic growth under selection in the presence of 1,3-propanediol, GEB028 (-B12) could grow in medium supplemented with 110g/l 1,3-propanediol. Analysis of aerobic growth of GEB031 on LB plates supplemented with 85, 95, 105 and 115g/l 1.3-propanediol showed that cells produce much bigger colonies in the presence of 85g/l in comparison with 105g/l. No growth was observed at 115g/l 1,3 propanediol. The results indicate that after 3 months of applying directed evolutions techniques described herein to *E. blattae*, the tolerance to 1,3 propanediol was increased from 75 g/l to at least 105 g/l under aerobic conditions. The plasmid was cured from the GEB031 strain by growing at 41.5 degrees. An illustrative clone, GEB031-4 was deposited with the ATCC and has accession number PTA-92.

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Please replace the paragraph beginning at page 26, lines 25-26, with the following rewritten paragraph:

*B5*

Strains - Wild type ATCC 33429, *E. blattae* comprising the mutant PDD as described in Example 4 and having ATCC accession number PTA-92.

Please replace Examples 6 and 7, beginning on page 27, line 30, through page 28, line 35, with the following:

Example 6: Cloning and sequencing the 1,3-propanediol dehydrogenase genes (*dhaT*) from *E. blattae*.

*gives a* The *dhaT* genes were amplified by PCR from genomic DNA from *E. blattae* as template DNA using synthetic primers (primer 1 and primer 2) based on the *K. pneumoniae* *dhaT* sequence and incorporating an *Xba*I site at the 5' end and a *Bam*HI site at the 3' end. The product was subcloned into pCR-Blunt II-TOPO (Invitrogen). The cloning *dhaT* were then sequenced with standard techniques.

*B6* The results of the DNA sequencing are given in SEQ ID NO:3 and SEQ ID NO:4.

Primer 1

5' TCTGATACGGGATCCTCAGAATGCCTGGCGGAAAAT3'      SEQ ID NO:14

Primer 2

5' GCGCCGTCTAGAATTATGAGCTATCGTATGTTGATTATCTG3'      SEQ ID NO:15

As will be readily understood by the skilled artisan, nucleic acid sequence generated via PCR methods may comprise inadvertent errors. The present invention also encompasses nucleic acid encoding PDD obtainable from *E. blattae* having ATCC accession number PTA-92.

Example 7: Comparison of wild-type *E. blattae* (ATCC accession number 33429) and the evolved strain GEB031-4 (ATCC accession number PTA-92).

This example shows that *E. blattae* subjected to the methods of the present invention and having ATCC accession number PTA-92 can completely consume 800mM glycerol during anaerobic fermentation and does not accumulate 3-hydroxy-propionaldehyde (3HPA) and does not lose viability. In contrast, the wild-type *E. blattae* accumulates 50mM 3 HPA and becomes non viable after consuming only 350 mM glycerol.

The wild-type *E. blattae* and the evolved *E. blattae* were subjected to fermentation in the following medium: 75 g glycerol, 5 g  $K_2HPO_4 \cdot 3H_2O$ , 3 g  $KH_2PO_4$ , 2 g  $(NH_4)_2SO_4$ , 0.4 g  $MgSO_4 \cdot 7H_2O$ , 0.2 g  $CaCl_2 \cdot 2H_2O$ , 4 mg  $CoCl_2 \cdot 2H_2O$ , 2 g yeast extract, and 1 g peptone per liter water. The pH was maintained with 20% NaOH. Both fermentations were run at 30°C with a  $N_2$  sparge and were inoculated with a stationary grown overnight preculture.--